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Mitochondrial DAMPS released by abdominal trauma suppress pulmonary immune responses

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Abstract

Objectives—Historically fever, pneumonia and sepsis after trauma are ascribed to pain and poor pulmonary toilet. No evidence supports that assertion however, and no known biologic mechanisms link injury to infection. Our studies show injured tissues release mitochondria (MT). Mitochondrial danger-associated molecular patterns (mtDAMPs) however, can mimic bacterial pathogen-associated danger molecules (PAMPs) and attract neutrophils (PMN). We hypothesized mtDAMPs from traumatized tissue divert neutrophils from the lung, causing susceptibility to infection.

Methods—Anesthetized rats (6–10/group) underwent pulmonary contusion (PC) by chest percussion. Modeling traumatic MT release, some rats had MT isolated from liver (equal to 5% liver necrosis) injected intra-peritoneal (IPMT). Negative controls had PC plus buffer IP. Positive controls underwent PC plus cecal ligation and puncture (CLP). At 16h bronchoalveolar and peritoneal lavages were performed. Bronchial and peritoneal lavage fluids (BALF, PLF) were assayed for PMN count, albumin, IL- β and CINC-1. Assays were normalized to BUN to calculate absolute concentrations.

Results—PC caused alveolar IL-1 β and CINC production and a 34-fold increase in BALF neutrophils. As expected, IPMT increased peritoneal IL-1 β and CINC and attracted PMN to the abdomen. But remarkably, IPMT after PC attenuated BALF cytokine accumulation and decreased BALF PMN. CLP had no direct effect on BALF PMNs, but like IPMT blunted BALF leukocytosis after PC.

Conclusions—Rather than acting as a 'second hit' to enhance PMN-mediated lung injury, mtDAMPs from trauma and PAMPs from peritoneal infection diminish PMN accumulation in contused lung. This may make the lung susceptible to pneumonia. This paradigm provides a novel

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CZ: Study design, literature search, data collection, data analysis, writing

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AG: Literature search, data interpretation, critical review of manuscript

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mechanistic model of the relationship between blunt tissue trauma, systemic inflammation and pneumonia that can be studied to improve trauma outcomes.

Keywords

Innate immunity; Neutrophils; DAMPs; Mitochondria; Trauma; Infection; Pneumonia

Trauma is the sixth leading cause of death worldwide, and is a serious public health problem with major social and economic costs. Local injury initiates an inflammatory cascade leading to local inflammation. More extensive injury can lead to systemic inflammation. Simultaneously though, immune-suppression develops and enhances the risk of infection in severely injured patients (1). Thus trauma patients are at high risk of infection both because physical barriers to tissue inoculation are disrupted and because of impaired host defense against bacterial inoculation (2,3) Thus infection is a leading cause of death after trauma. (2)

Blunt chest trauma is common and is an important contributor to 10% to 30% of adult trauma deaths. (4) Chest trauma is commonly accompanied by injury to other organ systems. Abdominal trauma is frequently associated with chest trauma, and is an important factor affecting mortality. (5) Pneumonia is the most common infection complicating chest trauma and is highly related to mortality. (6) Furthermore, pneumonia is the most common infectious complication in multiple trauma patients as a whole with an incidence reaching 30% after severe trauma. (7) Despite the critical association between chest trauma and chest infections in the trauma population, no basic biologic mechanisms have been advanced to explain the association of injury with pneumonia or the high morbidity and mortality of pneumonia after major trauma. Rather, associated findings like ‘splinting’ and ‘failure to breathe deeply’ have been causally implicated without rigorous scientific support.

Our previous studies have shown that cellular disruption by trauma releases mitochondrial debris (MTD) into the circulation that is rich in damage associated molecular patterns (DAMPs). These mitochondrial DAMPs show evolutionarily conserved similarities to bacterial Pathogen Associated Molecular Patterns (PAMPs). Thus they can initiate inflammation by signaling through innate immune pathways that create a sepsis-like state. (8–10) Sepsis however, does not necessarily lead directly to lung injury. (11) Another important phenomena we have observed is that is that MTD released by injured tissues attracts neutrophils. This is mediated at least in part by formyl peptides (FP) that act on formyl peptide receptors like FPR1. (10,12) Formyl peptides are typical of bacteria, but are also found in mitochondria. In addition to being neutrophil chemoattractants FP activate mononuclear cells releasing further chemokines and lipid chemoattractants. (13) We hypothesized that MTD released by injured tissue could suppress pulmonary immune responses by diverting neutrophils from the lung and or altering PMN function, thus potentially leaving the lung susceptible to infection

Materials and Methods

Hanks’ balanced salt solution (HBSS) was purchased from GIBCO (invitrogen, Grand Island, N.Y.), Heparin and 0.9% sodium chloride (saline) were purchased from Hospira

(Lake forest, IL), Phosphate buffered saline, Protease inhibitor cocktail and phosphatase inhibitor were purchased from Thermo Scientific (Rockford, IL). Other supplies were obtained as noted below.

Preparation of Mitochondrial Debris (MTD)

Mitochondria were isolated from rat liver acquired from male Sprague-Dawley rats (300–350g, Charles River) as previously reported. (10) Mitochondrial isolation kits for tissue (Thermo Scientific, Rockford, IL) were used to isolate liver mitochondria according to the protocol for Dounce homogenization of soft tissue supplied by the manufacturer. Mitochondria were isolated under sterile conditions at 4°C. Endotoxin levels were routinely measured by Limulus Amebocyte Lysate assay (Lonza, Walkersville, MD) and did not achieve significant levels. Isolated mitochondrial pellets were stored on ice pending further processing. Mitochondria were then suspended in Hanks Balanced Salt Solution (HBSS). Protease inhibitor cocktail (1:100) was added to the suspension, which was then subjected to sonication on ice using VCX130-Vibra Cell (Sonics and Materials, Newtown, CT) at 100% amplitude (5×, 30s each time with 30s intervals). Protein concentration of the suspension was determined by BCA assay (Thermo Scientific, Rockford, IL).

Animal protocols

All animal procedures were approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center (Boston, MA) in accordance with State, Federal and National Institutes of Health regulations. Anesthesia was induced using ketamine (40mg/kg) and xylazine (5mg/kg). Male Sprague-Dawley rats (Charles River, Boston, MA) weighing 200–250g were randomly divided into four groups: naïve controls, PC alone, MTIP and PC + MTIP. Naïve and PC only controls had injection of 1ml of HBSS buffer into the peritoneum. Meloxicam (1 mg/kg sc) was administered post-procedure for analgesia.

Pulmonary contusion (PC)

The pulmonary contusion model was adapted from the work of Badami and Livingston. This was developed to simulate the force generated in a 20 mph motor vehicular crash impacting a 70 kg man. (14) After anesthesia, rats were placed on a surgery board and restrained in the supine position. Animals were then subjected to a unilateral pulmonary contusion created with a Craftsman® EasyFire™ Forward Action™ Stapler. The stapler is fired directly against a 12.5g metal slug placed in a 2 cm plastic cup taped to the rat's right lateral chest. The injury is a clinically relevant, non-lethal hemorrhagic pulmonary contusion. (14)

Intra-peritoneal injection of rat mitochondria debris (IPMT)

Rats were restrained on a surgery board with the abdomen exposed. A 25G needle was inserted into the right lower quadrant, directed slightly cephalad and inserted 5 mm. Aspiration was used to insure that a hollow viscus had not been penetrated. The amount of MTD injected was equal to the mitochondria in 5% of the subject's liver (by weight) suspended in 1ml HBSS. When PC and IPMT were performed in the same rat, PC was performed first as described. All IP injections were performed under the same anesthesia.

Cecal ligation and Puncture

To model bacterial peritonitis, cecal ligation and puncture (CLP) controls were performed under anesthesia as previously described using a single puncture with a 21g needle. (15) These controls were done for the purposes of assessing the effect of an authentic abdominal infection on PMN mobilization to the lung after PC. Peritoneal lavage (PL) was not performed on CLP rats.

Sacrifice and removal of samples

15 hours after treatments (control, PC, IPMT and PC+IPMT), human serum albumin (HSA, 10 mg/kg) was injected via the tail vein. We chose this time point to model early onset pneumonia which is commonly seen after injury. 1 hour later, rats were anesthetized and the peritoneum was exposed. To actually perform PL, 5ml HBSS (containing 1% rat serum) and 3ml of air were injected and the rat was rolled and shaken. The peritoneum was then opened and lavage fluid was collected using a soft 23G needle. PL fluid (PLF) was centrifuged at 300×g for 10 minutes. Cell pellets were collected for PMN counting and supernatants were stored at -80°C. After PL, animals were exsanguinated by cardiac puncture. Blood was collected into minimally heparinized syringes and centrifuged at 3,000g for 10min at 4°C. Plasma aliquots were stored at -80°C. Finally, the lungs and trachea were excised and the trachea was cannulated. Bronchoalveolar lavage (BAL) was performed with saline (5ml lavage 5 times). BAL fluids (BALF) were centrifuged at 300g for 10 minutes to obtain cell pellets. PMN were counted by cytospin preparation using HEMA 3 stain (Fisher Scientific, Kalamazoo, MI). These supernatants aliquots were then also stored at -80°C.

Cytokine and chemokine ELISAs

All rat BALF, PLF and plasma samples were in aliquots frozen at -80°C. Experiments were performed in duplicate. HSA in rat BALF, PLF and plasma were measured using an ELISA Quantitation set specific for human albumin (BETHYL, Montgomery, TX). IL-1 β and CINC-1 in rat BALF or PLF were assayed using the Rat IL-1 β /IL-1F2 DuoSet and Rat CXCL1/CINC-1 DuoSet respectively (R&D, Minneapolis, MN). CINC-1 in rat plasma was determined using Rat CXCL1/CINC-1 Quantikine ELISA Kits (R&D, Minneapolis, MN). IL-1 β in rat plasma was assayed by Rat IL-1 β ELISA Kit (Thermo scientific, Rockford, IL).

Lung and peritoneal permeability assay

Human albumin (HSA) was measured in rat BALF, PLF and plasma to assess capillary leak using an ELISA quantitation set specific for HSA (BETHYL, Montgomery, TX). HSA is very similar to rat albumin in terms of size and diffusion, but we preferred to use HSA leak/hr as our readout for vascular permeability because we found it to be more reliable and accurate than direct rat albumin assays. Lung permeability was defined as the ratio of BALF to plasma HSA concentration and is reported as a percent. (16,17) Peritoneal permeability was similarly measured and reported as the ratio of PLF to plasma HSA concentration.

Urea concentration assay

We lavaged the tracheobronchial tree and peritoneum to obtain BAL and PL fluids. Thus the cytokines in the BALF and PLF were necessarily diluted to different degrees depending

upon buffer mixing with alveolar and peritoneal lining fluids as well as on possible absorption of the lavage fluid. To assess the absolute concentrations of chemoattractants at those sites for comparative purposes, we therefore normalized the diluted concentration results to urea concentrations, which are identical in plasma and all other extra-renal body fluids. Protein concentrations in BALF or PLF were then multiplied by the ratio of urea concentration in the plasma to that in the BALF or PLF to obtain the absolute protein concentrations in-vivo. Urea concentrations in the plasma, BALF and PLF were measured using the QuantiChrom™ micro-titer Urea Assay Kit (Bioassay systems, Hayward, CA).

Statistical analysis

Study data was assessed for statistical significance using Analysis of Variance (ANOVA) performed using SigmaPlot 11.0. Appropriate post-hoc tests were then chosen by the computer. All data are reported as mean $\pm$ S.E.M. with significances accepted at a $p < 0.05$.

Results

PMN migration to the lung (FIG 1A) and peritoneum (FIG 1B)

BALF PMNs were increased more than 30-fold by pulmonary contusion (PC). IPMT treatment had no independent effect on BALF PMN but markedly attenuated the ingress of PMN into the lung normally caused by injury (PC+IPMT, $P < 0.05$, ANOVA, Dunn's post hoc test). Conversely, PC had no independent effect on PMN in the peritoneum (Fig 1B). IP injection of MTD caused a significant influx of PMN into the abdomen (Fig 1B, column 3) that was not significantly altered by concurrent PC (Fig. 1B, column 4).

PMN migration to the lung after CLP (FIG 1C)

Infective peritoneal inflammation was created by CLP. CLP had no direct effect on BAL PMN counts. Similar to sterile inflammation from IPMT, CLP markedly decreased PMN ingress into the lung after PC ($P < 0.05$, ANOVA, Dunn's post hoc test). We did not measure PMN migration to the peritoneum after CLP.

Lung and peritoneal permeability (FIG 2A)

Permeability was measured as the percent leak of HSA from plasma into lung or peritoneum over 1 hour. Lung permeability was markedly increased by PC but not by IPMT. Unlike PMN influx, IPMT had little if any effect on the increase in permeability caused by PC (PC +IPMT). Conversely, IP injection of mitochondria significantly increased peritoneal permeability (Fig 2B, IPMT). PC had no significant effect on peritoneal permeability increase. It is noteworthy that lung permeability to albumin is generally an order of magnitude less than peritoneal permeability (Fig 2A vs Fig 2B, note scale differences).

IL-1 β levels in BALF, PLF and plasma

Macrophages are critical producers of IL-1 β and of CXC chemokines in response to all types of inflammation and so IL-1 β is a reliable marker for macrophage activation. (18,19) Here, we found that absolute IL-1 β levels (Fig. 3) were higher in lung than in the peritoneum or plasma. IL-1 β in BALF increased significantly after pulmonary contusion and peritoneal

IL-1 β levels increased after injection of IPMT. Conversely and again as generally expected, pulmonary contusion had little if any effect on peritoneal IL-1 β and IPMT had little effect on pulmonary IL-1 β . Statistically significant increases in plasma IL-1 β were seen after IP injection of mitochondria. But plasma concentrations were 5–10 fold lower than concentrations seen in tissues, so these variations are relatively small and unlikely to be biologically significant.

CINC-1 levels in BALF, PLF and plasma

The BALF assays in control rats showed that CINC-1 levels are normally much higher in pulmonary alveoli than in the peritoneum or the plasma (Fig. 4). Moreover, after pulmonary contusion, CINC-1 levels in BALF increased nearly 5-fold further ($P<0.05$), with CINC-1 levels in the BALF increasing to 36-fold simultaneous plasma levels. Injection of mitochondria into the peritoneum had no independent effect on BALF CINC-1 and attenuated the CINC increases seen in BALF after PC insignificantly (Fig. 4). CINC-1 levels were remarkably low in the peritoneum, again suggesting the relative specificity of CINC for PMN chemotaxis toward the lung. CINC levels in plasma only rose significantly ($P<0.05$) in animals sustaining both pulmonary contusion and intra-peritoneal MT. Plasma CINC concentrations were always about an order of magnitude lower than pulmonary concentrations. So these small changes in plasma CINC levels may simply represent 'spill-over' from inflammatory tissue sites. Of note however, the chemokine gradient from plasma to lung after PC (Figure 4) showed a more than 3-fold decrement when accompanied simultaneously by IPMT, decreasing from about 35-1 to about 10-1 ($P<0.05$).

Discussion

We began this study by defining specific mechanistic aspects of the inflammatory response to blunt chest trauma that could be followed after chest injury. Pulmonary contusion caused activation of local mononuclear cells, here indicated by elaboration of IL-1 β . Alveolar macrophages are critical producers of IL-1 β and also produce CXC chemokines in response to IL-1 β . (20,21) This we saw as robust increases in alveolar CXC-chemokines (eg. CINC-1). This was followed by PMN influx into the lung and increased alveolar permeability.

We then assessed the role of mtDAMPs (MTD) released by blunt liver injury by introducing relevant amounts MTD derived from rat liver into the peritoneum. This exposes peritoneal monocytes to MTD. We found that IP injection of MTD produced an abdominal environment that was strongly chemo-attractant for neutrophils (Fig 1B). PMN influx here might occur because mitochondrial formyl peptides attract PMN directly. In addition to formyl peptides however, mitochondria contain numerous other alarmin motifs (like mtDNA) that can activate production of other chemoattractants by monocytes (suggested by IL-1 β release, Fig 3C) or directly by PMN thus creating an inflammatory environment. Thus the data show release of mitochondrial DAMPs at injury sites in the periphery can induce local chemoattractant environments that can divert PMN from the lung, potentially making the host more susceptible to pneumonia. We also saw that sterile mtDAMPs created very similar responses to those seen when CLP-derived pathogens entered the peritoneum.

Two decades ago Antonelli et al. (22) showed in clinical trauma patients that the strongest risk factor for early onset pneumonia was a combination of abdominal and thoracic trauma. This increased risk of pneumonia by eleven fold. The known association between abdominal injury and early onset pneumonia was a key reason that we chose to study the mechanisms underlying pneumonia at an early time point after injury. During the past decade, a number of experimental and clinical studies have indicated that trauma profoundly affects immune responses, leading to some form of immune paralysis. (23–26) PMNs are prototypical phagocytes and play a central role in host innate immune responses. Here we show clearly that one mechanism by which the PMN response, critical to innate protective immunity against pulmonary infection, can be suppressed by tissue injury. Thus our mechanistic studies parallel the clinical findings of Antonelli et al. (22). Recruitment and activation of PMN requires the presence of chemokine gradients and the ability of PMN to respond to them appropriately. We have previously found these responses to be blunted after injury *in-vivo* (27) as well as by the actions of MTD on PMN formyl peptide receptors in native cells *in-vitro*. (10) We now show that systemic accumulations of mtDAMPs in tissue, as will occur in injury, can lead to impaired PMN function. CINC is the homologue of GRO- α , a primary PMN-specific chemokine. (28) Changes in CINC-1 concentration will be especially relevant to PMN function when they occur in the region of the EC₅₀ of CXC receptors, as we found here. (28,29) Thus in this model, we see modest changes in chemokine gradients from plasma to lung (Fig. 4) are associated with important changes in PMN recruitment to the lung. This may reflect changes in relative gradients from lung to plasma (Fig 4). There we saw that after contusion, CINC gradient from lung to plasma rose from 10:1 to 36:1. IPMT had no effect on gradients per se but after PC+IPMT, CINC gradients from lung to plasma fell back to 10:1, approximating naïve values.

Impaired PMN trafficking to the lung after injury might in fact depend on several DAMP-related mechanisms. First, formyl peptides (FP) of either bacterial or mitochondrial origin are potent direct chemoattractants, acting on PMN via Formyl Peptide Receptor-1 (FPR1). Chemotaxis to sites of FP accumulation might therefore act as a systemic ‘sump’ for PMN, diverting them away from the lung simply by virtue of numeric competition. We saw the same effect when authentic bacterial infection was created using CLP (Fig. 1C). Others have found similar patterns of PMN diversion from the lung in the face of peritoneal infection. (30) Second, CINC-1 is the rat homologue of the prototypic human CXC-chemokine GRO- α (CXCL1). CXC-chemokines play a critical role in PMN migration toward inflammatory sites and in subsequent PMN activation. (31–35) PMN exposure to FP in mtDAMPs however, induces down-regulation of the high-affinity CXC receptor CXCR2 (10) as we have also shown to occur *in-vivo* after clinical trauma. (36) Thus local mtDAMPs at injury sites may activate chemotaxis toward injury as well as directly suppress chemotaxis toward the lung.

In summary, these studies show that pulmonary injury causes PMN migrate to lung and that mitochondria given IP can simulate abdominal trauma, causing PMN to migrate to the abdominal cavity. When these two challenges occurred simultaneously, rather than IPMT acting (as we initially hypothesized) as a synergistic stimulus for PMN attack on the lung, it diverted PMN from the lungs. This paradigm might paradoxically seem beneficial where

pulmonary PMN chemotaxis to the lung is of concern only with respect to the genesis of acute lung injury. But the primary reason for PMN migration to the lung is control of the constant bacterial inoculation of the lung that occurs in the poly-traumatized patient. These study data indicate that when mitochondria are released from injured, dying or dead tissue, PMN that would normally respond to infective pulmonary stimuli can be diverted to peripheral sites of injury. We predict that this will make the lung susceptible to infection and studies are now under way to test this hypothesis. This novel mechanistic paradigm may explain the clinical relationship between systemic blunt tissue injury, pneumonia and sepsis.

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Fig. 1. Neutrophil trafficking

A) PMN in BALF of control rats, rats with a pulmonary contusion (PC), rats injected with intra-peritoneal mitochondria (IPMT) and rats subjected to PC and IPMT (PC+IPMT). PC increased BALF PMN by >30 fold. IPMT had no direct effect on BALF PMNs but markedly attenuated PMN entry into the lung after PC. $n=8-10/\text{group}$, * denotes $P<0.05$ vs control, † means $P<0.05$ versus PC. **B)** PMN in peritoneal lavage fluids (PLF): PC has no effect on PMN migration to the peritoneum. IPMT causes significant increases in PLF PMN with or without PC. **C)** Cecal ligation and puncture (CLP) was used as a positive control to

compare the effects of intraperitoneal MT to an “authentic” infection. Once again, we see the presence of “danger” signals (here infectious rather than sterile motifs) diminishes PMN trafficking to the injured lung. * denotes $P < 0.01$ vs control.

§ signifies $P < 0.05$ for PC+CLP vs PC only. (All testing is by ANOVA/Dunn’s test).

Fig. 2. Permeability changes

A) Lung permeability: pulmonary contusion (PC) tripled lung albumin leak ($P < 0.01$). IPMT had no significant direct effects on lung leak or on lung permeability caused by the PC. **B) Peritoneal permeability:** IPMT (5% of the liver's MT mass) significantly increased peritoneal permeability as compared with control conditions. PC *per se* did not cause significant peritoneal permeability. Concurrent PC had no effect on the increase in peritoneal permeability caused by IPMT. (n=6–8/group, *denotes $P < 0.01$ vs control. \$ denotes $P < 0.05$ vs IPMT, ANOVA/Dunn)

Fig. 3. IL-1 β levels in plasma, BALF and PLF

(A) IL-1 β level in plasma. Compared with control group, [IL-1 β] in MTIP group and PC +5%MTD IP group increased significantly (n=5–8). *denotes $P < 0.05$ versus control (ANOVA/ Tukey). (B) IL-1 β level in BALF. Compare with control group and 5% MTD IP group, [IL-1 β] in PC group increased significantly. [IL-1 β] in 5%MTD IP +PC group didn't increased too much, it was lower than PC group. (n=5–8, *denote $P < 0.05$ versus control, \$ denotes $P < 0.05$ versus 5%MTD IP group, ANOVA/ Tukey). (C). IL-1 β level in PLF. Compare with control group and PC group, [IL-1 β] in 5%MTD IP group increased significantly (n=6–8, *denote $P < 0.05$ versus control, ANOVA/ Dunn's). (D) IL-1 β level in plasma, BALF and PLF (pg/ml). (E) Percentage of IL-1 β in BALF and PLF (% IL-1 β in plasma)

Fig. 4. CINC-1 levels in plasma, BALF and PLF

CINC levels were uniformly much higher in the lung than in other locations. CINC-1 in BALF increased significantly after PC (*signifies $P < 0.05$ vs. control BALF). After IPMT lung CINC response to PC was less but not significantly so. A modest but significant increase in plasma CINC was also noted when there was both lung injury and peritoneal inflammation († denotes $P < 0.05$ versus control plasma). But the enhancement of CINC *gradient* from lung to plasma by PC was therefore significantly diminished by IPMT, changing from an approximately 35-fold gradient to a 10-fold gradient ($P < 0.05$ ANOVA/Dunn's).